Video Article

Custom-designed Laser-based Heating Apparatus for Triggered Release of Cisplatin from Thermosensitive Liposomes with Magnetic Resonance Image Guidance

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Abstract

Liposomes have been employed as drug delivery systems to target solid tumors through exploitation of the enhanced permeability and retention (EPR) effect resulting in significant reductions in systemic toxicity. Nonetheless, insufficient release of encapsulated drug from liposomes has limited their clinical efficacy. Temperature-sensitive liposomes have been engineered to provide site-specific release of drug in order to overcome the problem of limited tumor drug bioavailability. Our lab has designed and developed a heat-activated thermosensitive liposome formulation of cisplatin (CDDP), known as HTLC, to provide triggered release of CDDP at solid tumors. Heat-activated delivery *in vivo* was achieved in murine models using a custom-built laser-based heating apparatus that provides a conformal heating pattern at the tumor site as confirmed by MR thermometry (MRT). A fiber optic temperature monitoring device was used to measure the temperature in real-time during the entire heating period with online adjustment of heat delivery by alternating the laser power. Drug delivery was optimized under magnetic resonance (MR) image guidance by co-encapsulation of an MR contrast agent (*i.e.*, gadoteridol) along with CDDP into the thermosensitive liposomes as a means to validate the heating protocol and to assess tumor accumulation. The heating protocol consisted of a preheating period of 5 min prior to administration of HTLC and 20 min heating post-injection. This heating protocol resulted in effective release of the encapsulated agents with the highest MR signal change observed in the heated tumor in comparison to the unheated tumor and muscle. This study demonstrated the successful application of the laser-based heating apparatus for preclinical thermosensitive liposome development and the importance of MR-guided validation of the heating protocol for optimization of drug delivery.

Video Link

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Introduction

The pathophysiology of solid tumors results in the enhanced permeability and retention (EPR) of nanoscale systems. This has led to the development of many drug delivery systems that take advantage of this effect to target the tumor tissue while minimizing systemic side effects¹. Liposomal delivery technologies have been widely investigated for drug or imaging probes². Although liposomes have significantly reduced the systemic toxicity compared to conventional chemotherapy, there have been few improvements in clinical efficacy^{3,4}. Studies have shown that the limited efficacy is due to a lack of drug release from the carrier^{4,5}. As a result, development of liposomes that are activated to release the encapsulated drug in response to external stimuli has attracted considerable attention. Hyperthermia has been employed for decades as a relatively safe treatment modality for cancer patients⁶. Therefore the development of thermosensitive liposomes with heat as an external trigger has been a logical combination with significant potential for clinical translation. Indeed, the lysolipid-containing thermosensitive liposome formulation of doxorubicin, known as LTSL-DOX, has now reached clinical evaluation⁷.

Recent clinical data with LTSL-DOX has shown that the protocol for heat delivery is a critical factor that can heavily influence patient outcomes⁸. In humans, radiofrequency, microwave, laser and ultrasound transducers are used to apply hyperthermia locally at tumor sites⁹. In preclinical studies requiring heating of subcutaneous tumors, heating catheters^{10,11} and water baths^{12,13} are most often employed. In this manuscript, we introduce a new method for heating subcutaneous tumors using a custom designed laser-based heating setup, which enables more conformal

heating of the tumor volume. Using MR compatible materials, the setup is small enough to fit within the bore of a small animal MR imager, allowing real time monitoring of changes in tissue temperature during the laser heating.

The MR contrast agent, gadoteridol (Gd-HP-DO3A), was co-encapsulated with CDDP into a thermosensitive liposome formulation of CDDP (HTLC), known as Gd-HTLC, for real-time MR image-guided monitoring and assessment of heat-activated drug release and validation of the heating protocol. Our results demonstrate that the laser-based heating apparatus efficiently activated the release of encapsulated agents from the Gd-HTLC formulation while being monitored through MR imaging.

Protocol

1. Liposome Preparation

1. Dissolve the lipids 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (MSPC or S-lyso-PC) and *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (mPEG₂₀₀₀-DSPE) in chloroform. For example, for preparation of 10 ml of HTLC, weigh out 314.4 mg DPPC, 39.4 mg MSPC, and 83.9 mg mPEG₂₀₀₀-DSPE into an amber glass vial. Then, dissolve the lipids in 2 ml of chloroform and heat up the vial for 30 sec in a 60 °C water bath.
2. Remove the chloroform using a rotary evaporator. Place the resulting lipid film under high-pressure vacuum O/N.
3. For 10 ml of HTLC, hydrate the lipid film with 5 ml 0.1N Tris buffer (pH 7.4) for 1 hr. At the same time, weigh out 162.4 mg 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) lipid and 100 mg CDDP powder and hydrate with 5 ml 0.1N Tris buffer containing 30% ethanol (pH 7.4) for 1 hr.
4. For the Gd-HTLC formulation, weigh out the same amount of DPPG lipid and 10 mg CDDP powder, then hydrate with 2.5 ml Gd-HP-DO3A solution (279.3 mg/ml) plus 2.5 ml 0.1N Tris buffer containing 30% ethanol (pH 7.4) for 1 hr. During hydration, the mixtures must be kept in amber vials and placed on a hotplate at 70 °C with constant stirring and vortexing every 10 min.
5. Combine the lipid mixture and the lipid drug mixture, and again hydrate for 1 hr. Vortex every 15-20 min.
6. Assemble the 10-ml extruder with two stacks of 200 nm polycarbonate filters. Connect the thermobarrel of the extruder to a circulating water bath at 70 °C, and connect the extruder to a compressed nitrogen tank.
7. Immediately after hydration, transfer the mixture into the extruder chamber. Open the nitrogen flow (set pressure to 200 psi) to extrude the liposomes through the membranes. Collect the liposomes in a 50 ml tube. Keep the tube in a hot water bath (70 °C) at all times during the extrusion process. Repeat this process 5 times.
8. Disassemble the extruder and change to two stacks of 100 nm polycarbonate filters. Reassemble the extruder and set the pressure to 400 psi. Repeat Step 5, except extrude the liposomes 10 times. Collect the sample from the final extrusion into a 15 ml tube.
9. Cool down the liposomes to RT, and centrifuge at 1,000 x g for 3 min to precipitate insoluble CDDP.
10. Dialyze the liposomes O/N against 0.9% saline in dialysis tubing with a 15,000 molecular weight cutoff (MWCO) under sterile conditions.
11. Dilute 10 µl of the liposomes in 990 µl distilled water. Use dynamic light scattering to measure the size distribution of HTLC and Gd-HTLC. Perform 3 measurements with 10 runs each.
12. Dilute 40 µl of the liposomes in 3,960 µl distilled water. Make 5-6 standard solutions of platinum and gadolinium in the concentration range of 0.1 to 20 µg/ml. Measure the platinum and gadolinium concentrations using inductively coupled plasma-atomic emission spectrometer (ICP-AES) at 3 different wavelengths. Perform 3 measurements at each wavelength for average result.
13. Determine the gel to liquid crystalline phase transition temperature (T_m) of the HTLC liposome formulation using a differential scanning calorimeter (DSC). Load 5-10 mg of HTLC into a DSC pan and use an empty pan as a reference. Use a scan rate of 5 °C/min to ramp up the temperature from 0 °C to 60 °C.
14. Wrap the liposomes in aluminum foil to prevent light exposure and store at 4 °C.

2. In vitro Release from Liposomes

1. Prepare spin columns.
  1. Incubate 50 g gel filtration beads in 400 ml 0.9% saline at RT for 3-4 hr. Roll up a piece of glass wool, wet with 0.9% saline and place it into the tip of a 1 ml syringe. Press the glass wool to fill approximately 0.05-0.1 ml of the syringe. Use a glass pipette to gradually add about 1 ml gel filtration media into the syringe.
  2. Place the syringe into a 15 ml tube and centrifuge at 1,000 x g for 3 min. Remove the spin column and place it into a 15 ml tube.
2. Sample 400 µl of HTLC or Gd-HTLC into 8 1-dram glass vials and incubate in a temperature controlled water bath at either 37 °C or 42 °C. Take out one vial at each time point (i.e., 5, 15, 30 and 60 min) and immediately place it on ice.
3. Add 100 µl of 0.9% saline into the prepared spin column, then add 100 µl of HTLC or Gd-HTLC before incubation (control) or after incubation in the water bath into the spin column. Centrifuge at 1,000 x g for 3 min. Remove the syringe from the tube and dilute the solution in the tube for ICP-AES analysis.

3. Implantation of Subcutaneous Xenograft of Cervical Tumor

1. All animal studies were conducted according to animal use protocols approved by the Animal Care Committee of the University Health Network (UHN).
2. Perform all animal studies in a Biosafety Cabinet (BSC). Autoclave all surgical tools before any surgery. After each surgery, wipe the surgical tools with 70% ethanol and sterilize again using a hot bead sterilizer.
3. For euthanasia, place the animal in an insulated CO₂ chamber, then perform cervical dislocation.
4. For general anesthesia using 100% oxygen, use 5% isoflurane for induction and 2% for maintenance. To ensure the depth of anesthesia, apply pressure with forceps to palmar surface of the footpad to observe animal response. Apply eye lubricant to prevent dryness while under anesthesia.

5. For an animal that has undergone surgery, place the animal in a clean cage without the company of other animals. Monitor until sufficient consciousness is regained.
6. Culture the ME-180 cells in alpha-minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.
7. Harvest the cells and keep the cells in complete media before inoculation. Count the cells using a hemocytometer.
8. Inoculate each donor mouse to bear an intramuscular (i.m.) ME-180 (human cervical cancer cells) tumor.
Note: Growth at the i.m. site has been selected in order to ensure the development of well vascularized tumors. Purchase female SCID mice (aged 6-8 weeks, approximately 20 g) from an in-house breeding facility.
  1. Anesthetize a female SCID mouse and inject 1×10^6 ME-180 cells into the gastrocnemius muscle of the hind limb using a 27 G needle.
  2. Measure tumor size using a caliper until the tumors have reached 9-12 mm in their longest dimension. Terminate the study if the tumor has exceeded 12 mm, or if the tumor mass compromises normal behavior, ambulation, food and water intake.
9. Implant tumor pieces from donor mice into recipient mice.
  1. Anesthetize a donor mouse using 5% isofluorane. Euthanize the mouse using cervical dislocation under anesthesia. Remove the donor tumor from the donor mouse. Cut the tumor into cubic fragments of 2-3 mm³.
  2. Anesthetize a recipient mouse using 5% isofluorane. Inject 100 μ l of 0.5 mg/ml meloxicam subcutaneously before surgery. Apply iodine surgical scrub solution, then 70% ethanol, and lastly iodine solution to the shaved skin. Shave the left hind limb of the recipient mouse. Make an incision at the level of skin. Insert one donor tumor piece subcutaneously through the incision. Close the incision using 1-2 wound clip(s).
  3. Remove the clips 3-5 days after implantation. Subcutaneous tumor is required for using the laser-based heating setup.
  4. Allow tumors to grow for 2-3 weeks before heating treatment.

4. Design, Assembly and Calibration of a Conformal Laser Delivery Illuminator for *In Vivo* Heating

1. Achieve tissue heating using a 763 nm diode laser coupled to a conformal illuminator using an optical fiber.
  1. The illuminator provides uniform superficial laser illumination of the xenograft tumor. It is composed of a 30 x 20 x 17 mm block of highly reflective material containing three adjoined light integrating chamber spheres with one chamber in the middle (16 mm in diameter) and 2 small chambers on either side (16 mm in length and 5 mm in diameter) (**Figure 1**).
  2. In order for light to pass from one chamber into the other, two small 5 mm diameter holes were cut between the small outer chambers and the larger middle chamber. Light is delivered from the laser into the outer chambers using a 400 μ m cut-end fiber connected to the laser that is passed into the chamber through a 600 μ m diameter hole.
  3. Due to the nature of light interaction with the chamber walls, light delivered into one of the small chambers is spatially homogenized then passes through the interior ports into the larger chamber where it is further spatially homogenized. Light then exits the 10 mm diameter port on the middle chamber.
2. Calibrate the illuminator light delivery with respect to the laser power setting using a NIST calibrated 50 mm integrating sphere to measure the delivered power.
  1. Calculate the light distribution in the tumor using Monte Carlo simulations based on a simplified geometry of the tumor. Generate the Monte Carlo code in a custom algorithm written with a commercial computational software package and base on the standard code of Monte Carlo modeling of light transport in multi-layered tissues of Jacques *et al.*¹⁴.
  2. Model the tumor as a hemisphere lying on a flat skin line with a diameter matching the average tumor dimension of 7 mm at the skin surface and a height of 5 mm. Model homogeneous illumination of the surface by randomly launching photons throughout the exposed hemisphere and direct at the center of the sphere.
  3. Use optical properties including absorption, $\mu_a = 0.025 \text{ mm}^{-1}$, scattering, $\mu_s = 10 \text{ mm}^{-1}$, anisotropy factor, $g = 0.9$ and refractive index, $n=1.4$. Use one million photons in the calculation.
  4. Perform these measurements before any *in vivo* heating experiments with no further modifications after initial calibration.

5. Conformal Heating of Tumor using Custom-designed Laser Chamber Setup

1. Connect one end of a laser fiber to the laser device and the other end to the illuminator.
2. Anesthetize the animal using 5% isofluorane. Insert a 27 G injection catheter into the lateral tail vein of the mouse. Insert a 22 G catheter into the center of the tumor. Place a fiber optic temperature probe into the hollow catheter to monitor temperature change.
3. Cover the entire tumor with the illuminator (**Figure 2**). First set the power to 0.8-1 W. Turn on the laser and wait for the temperature to rise. Maintain the temperature of the tumor at 42 °C by manually adjusting the laser power between 0.1-0.8 W.

6. Temperature Distribution Evaluated Through MR Thermometry (MRT)

1. Measure the temperature distribution of the heated tumor using the laser-based heating setup through proton resonance frequency shift (PRF-shift) MRT on a 7 Tesla preclinical MR imaging system¹⁵ in conjunction with the fiber optic temperature probe measurements.
2. Acquire thermometry images at 10 sec intervals using a 2D-FLASH pulse sequence (echo time 4.5 msec; repetition time 156.25 msec) with 312 x 312 μ m in-plane resolution and 2 mm slice thickness in a single slice at the level of the fiber optic temperature probe.

7. MR Monitoring of Agent Release

1. Perform T₁-weighted imaging prior to and 20 min after administration of Gd-HTLC.

2. Implant two tumors, one on each of the hind limbs, of a female SCID mouse using the method described above in Section 3 for the implantation. Preheat the tumor on the left hind limb for 5 min at 42 °C prior to injection of Gd-HTLC at a dose of 66.3 mg/kg Gd-HP-DO3A and 1.4 mg/kg CDDP, then heat the tumor for an additional 20 min post-injection. Perform injection during heating treatment. Use the tumor on the right hind limb as an unheated control.
3. Acquire dynamic MR images (36 x 20 mm field-of-view, 200 x 200 μ m in-plane resolution, 2 mm slice thickness; MR sequence protocol) to monitor Gd-HP-DO3A release at 12 sec intervals for the entire 20 min post administration of Gd-HTLC, beginning 30 sec prior to injection.
4. Contour the tumor (heated and unheated) and muscle volumes. Calculate the mean MR signal of all voxels within each contoured volume.

Representative Results

The HTLC liposomes are manufactured using common methods, including lipid film formation, hydration, extrusion and dialysis. During steps involving CDDP, caution should be taken not to expose CDDP to any aluminum material, as CDDP will be deactivated through the formation of a black deposit. An illustration of HTLC is shown in **Figure 3**. The physico-chemical properties of HTLC were summarized in a manuscript recently published in the *Journal of Controlled Release*¹⁶. The gadolinium and platinum concentrations of the Gd-HTLC formulation are 1.87 $\pm$ 0.28 mg/ml and 0.10 $\pm$ 0.02 mg/ml, respectively.

The illuminator of the laser-based heating setup uses three small, connected chambers that provide a homogeneous light distribution ($\pm$ 15%) at the 10 mm diameter exit port. Depending on the power setting of the laser, power is delivered within the range of 0.5 to 1.7 W/cm², as measured using a calibrated integrating sphere. The results are shown in **Figure 4** as a cross-section through the tumor. Assuming a total power of 1 W, the maximum fluence rate at any point in the tumor is 70 mW/cm². The laser power decreases by a factor of 2 at the skin level compared to the peak fluence just below the tumor surface.

Heating using the laser-based heating setup generates a relatively uniform temperature distribution map, as confirmed by the PRF-shift MRT (**Figure 5**). PRF-shift MRT tracks the relative temperature change from an absolute baseline measurement acquired before heating by the point source (*i.e.*, fiber optic temperature sensor).

From MR signal analysis (**Figure 6C**), the heated tumor displays the highest relative signal increase in comparison to the unheated tumor and muscle after administration of Gd-HTLC, which is maintained until the end of the heating period.

Figure 1. Design of the illuminator. (A) Dimensions of the illuminator. **(B)** Dimensions of the inner chambers. [Please click here to view a larger version of this figure.](#)

Figure 2. Illustration of the laser-based heating setup along with a temperature monitoring device. A laser fiber (blue line) delivers light to the illuminator. A fiber optic temperature probe (yellow line) was placed into the center of the tumor through a 22 G catheter to monitor temperature change. Real-time temperature readings are shown on the computer screen. Modified from *Journal of Controlled Release* 2014, 178, 69-78.¹⁶ [Please click here to view a larger version of this figure.](#)

Figure 3. Schematic drawing of the HTLC formulation (not to scale). The lipid compositions, CDDP concentration and size of the HTLC liposomes are illustrated. Modified from *Journal of Controlled Release* 2014, 178, 69-78.¹⁶ [Please click here to view a larger version of this figure.](#)

Figure 4. Modeling of light delivery to tumor using small integrating sphere. (A) Schematic of model showing tumor raised above the normal underlying tissue. Red arrows represent coverage and direction of initial photons in the calculation. Illumination covers the full surface area of the raised tumor. **(B)** Results of calculation showing light fluence distribution in the tumor. **(C)** Cross-sectional plot of light fluence versus depth, along the white dashed line shown in **(B)**. Fluence rate in the tumor at the skin depth is 50% of the maximum fluence rate. [Please click here to view a larger version of this figure.](#)

Figure 5. Temperature distribution evaluated through MR thermometry. (A) T₂-weighted image showing the anatomical location of the two bilaterally implanted tumors. **(B)** Temperature distribution map generated from heating the left tumor to 42 °C using the laser-based heating setup, while the right tumor remained unheated. Data re-analyzed from *Journal of Controlled Release* 2014, 178, 69-78.¹⁶ [Please click here to view a larger version of this figure.](#)

Figure 6. MR monitoring of gadoteridol release from the Gd-HTLC liposomes upon heat activation. T₁-weighted MR images (same window level applied) of a mouse bearing two subcutaneous ME-180 tumors, with one on each of the hind limbs (A) pre-injection of Gd-HTLC and (B) 20 min post-injection of Gd-HTLC (i.e., after the entire heating period). (C) Relative MR signal changes normalized to the first time point of the dynamic MR acquisition of the mouse shown in (A) and (B). Data represent mean + SD. Data re-analyzed from *Journal of Controlled Release* 2014, 178, 69-78.¹⁶ Please click here to view a larger version of this figure.

Discussion

Liposomes were first developed in the 1960s as drug delivery vehicles that carry hydrophilic drugs in their internal aqueous volume and hydrophobic drugs within their lipid bilayer². In addition to use in therapeutic applications, liposomes have been explored for diagnostic applications when labeled with radionuclides or loaded with imaging contrast agents¹⁷. In recent years, theranostics and therapeutic-diagnostic pairs have been pursued to provide opportunities for image-guided patient stratification and drug delivery^{17,18}. The current study builds on the concept of image-guided drug delivery to evaluate triggered drug release from thermosensitive liposomes using a custom-designed laser-based heating setup under MR guidance.

As mentioned above, water baths or heating catheters have commonly been used to heat subcutaneous tumors. The water bath method requires the immersion of the entire limb in hot water, resulting in non-specific drug release, throughout the limb in addition to release at the tumor site. Use of a heating catheter requires placement of an 18 G catheter in the center of the tumor, and has been shown to necessitate heating for a relatively long period of time (15 min) in order to reach thermal steady state¹¹.

In the present study, the newly designed laser-based heating setup provides a conformal way of delivering heat to the tumor volume as demonstrated by MR-based assessment of the temperature distribution map (Figure 5). Heterogeneity in the PRF-shift map in the unheated

right hind limb and right limb tumor in **Figure 5** may reflect a combination of low signal-to-noise in the vicinity of susceptibility artifacts and slight physiological or heating-induced motions, which may directly compromise heating and baseline image registration but also introduce slight variations in induced fields around regions of offset magnetic susceptibility. In addition, it was found that thermal steady state could be achieved within 1-2 min of initiating heating. Also, the point-based fiber optic temperature sensor allowed for real-time adjustment of the laser power in order to maintain the temperature at 42 °C and to minimize temperature fluctuations. However, it is critical to place the fiber optic temperature sensor in a relatively central position within the tumor. MR imaging can be employed to validate the incision point for the sensor. During heating, the laser power should be carefully adjusted to maintain the temperature at 42 °C with an initial power of 0.8 W.

The heating protocol was optimized through real-time monitoring of Gd-HP-DO3A release as a surrogate for the drug CDDP. The effective release of encapsulated agents from the HTLC liposomes translated into improved efficacy, with the heated HTLC group resulting in a significant therapeutic advantage over other treatment and control groups¹⁶.

The illuminator of the heating apparatus was designed to heat tumors of 5-7 mm in the largest dimension. The design of the illuminator could be modified to heat larger tumors and multiple fiber optic temperature sensors could be inserted throughout the tumor volume to monitor temperature in comparison to the current single-point based measurement. In addition, since the portable device is made from MR-compatible materials, 3-dimensional MR thermometry could be performed to evaluate the temperature distribution throughout the entire tumor volume.

In conclusion, the laser-based heating apparatus provides a valuable tool for preclinical development of thermosensitive liposome formulations. Image-guided drug delivery approaches, such as the one used in this study, have significant potential for clinical translation and implementation of personalized medicine including real-time monitoring of therapeutic treatment.

Disclosures

There are no disclosures.

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